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Morpho- Physiological and Biochemical Evaluation of Two *Indica* Rice (*Oryza sativa* L.) Cultivars under Abiotic Stresses at the Seedling Stage

Karikalan Jayaraman[1, 2], Amitha Mithra Sevanthi[1], Sivakumar S.R.[2], Kiran Babu P.[3], Pranab Kumar Mandal[1*]

[1]ICAR-National Institute for Plant Biotechnology, Pusa Campus, New Delhi, India; [2]Department of Botany, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India; [3]ICAR-Indian Institute of Oil Palm Research, Pedavegi, Andhra Pradesh, India

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Abstract

Rice provides staple food for a large part of the world's human population. Abiotic stresses, such as salinity, heat and low temperature stimulates diverse morphological, physiological and biochemical responses that negatively impact the plant growth, development and productivity of crops. Screening of rice genotypes against abiotic stress is a fundamental step to understand the mechanism and stress tolerance of rice cultivars. Two indica rice cv Nagina22 (N22) and Pusa Sugandh2 (PS2) were screened for salt (150mM NaCl), cold (12°C) and heat (40°C) stress responses through morpho-physiological and biochemical traits at the seedling stage. FL478 and MTU1010 varieties were used as salt and cold tolerant control, respectively. The present investigation showed salinity and cold tolerance of PS2 was noticeable through lower reduction in fresh and dry weight of seedlings, photosynthetic pigments and associated with higher accumulation of proline, lower ion leakage and MDA content under salinity and cold stresses. In contrast, under high temperature treatment, PS2 genotype had significantly higher reduction of biomass and photosynthetic pigments, less cell membrane stability, higher lipid peroxidation, and lower proline accumulation as compared to N22. From our study, PS2 was found to be tolerant to cold and salinity stress, while N22 was found to be tolerant to heat stress. Evaluation of abiotic stress tolerance at seedling stage against salt, cold and heat stresses by utilizing morphological, physiological and biochemical approaches is an appropriate way in rice, when used along with suitable check variety.

Keywords: Rice, Salinity, Cold, Heat, MDA Content, Electrolyte Leakage, Proline, Photosynthetic Pigments.

Corresponding author's e-mail: pranabkumarmandal@gmail.com
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Introduction

Rice is the second most important crop among cereals after wheat that provide staple food for a large part of the world's human population, especially in Asia (Muthayya et al., 2014). Various abiotic stresses such as drought, salinity, heat and low temperature are the most common environmental factors, which negatively impact the growth, development and productivity of crops (Scafaro et al., 2011). In order to overcome these limitations, there is an urgent need to develop crop varieties tolerant to such abiotic stresses. The screening of rice plants at the seedling stage is commonly acceptable as it provides a rapid screening method, while it is difficult at the vegetative and reproductive stages (Gregorio et al., 1997).

Salinity is the second important abiotic stresses in rice after drought stress in the developing countries. Approximately one billion hectares of world's total land area has been affected by soil salinity (Fageria et al., 2012). To improve salinity tolerance in rice varieties, it is very important to find adequate variation and to devise proper screening techniques which are reliable and able to identify salt tolerant varieties (Kranto et al., 2016). Rice is a salt sensitive crop; although salinity stress affects all stages of the growth and development of rice, seedling and reproductive stages are more sensitive (Zeng et al., 2001; Ali et al., 2014). The study of Garg et al (1996) reported that seedlings survival and growth was important morphological index to identify the salt tolerance of a cultivar. The root growth was more affected than shoot growth in the seedling stage under salt stress in rice (Hakim et al., 2010; Kumari et al., 2016).

Heat stress has become a serious problem of agriculture in various regions worldwide due to global warming (Zafar et al., 2017). Exposure of crops to heat stress is likely to increase with the projected increase in the global average surface temperature of 2.0-4.5°C (IPCC, 2007). Rice productivity would be reduced 4.1-10% due to the seasonal average temperature increased by 1°C (Peng et al., 2004). Heat stress inhibit seedling establishment leading to non-uniform growth and reduced the yield under the early stage of growth in rice (Lei et al., 2013; Zafar et al., 2017). Cold is a problem in rice production during winter season in India. Cold tolerance in rice can be illustrated as the ability to germinate, emerge and grow under low temperatures ranging from 6-15°C. The 3^{rd}-4^{th} leaf stage of rice plant is the most sensitive stage to low temperature. Rice cultivation is negatively affected by cold stress during seedling stage resulting in poor germination, stunted seedling growth yellowing and ultimately seedling mortality, besides the fungal attack which is commonly associated with cold stress (Pathak et al., 2003; Khush and Jena, 2009).

Abiotic stress elicits various morphological, physiological and biochemical responses in rice plants so as to acclimatize them for survival under such harsh environmental stress condition. Abiotic stress adversely affects reduction in fresh and dry weight of seedlings and photosynthetic pigments, such as chlorophyll a, b and carotenoids in many plant species (Bonnecarrere et al., 2011; Hameed et al., 2012; Rasel et al., 2020). In different crop plants, proline as osmo-protectant and cryo-protectant (Yoshiba et al., 1997), cell membrane stability for evaluating potential abiotic stress tolerance (Hameed et al., 2012; Zafar et al., 2015) electrolyte leakage and Malondialdehyde (MDA) content, specifically for salinity tolerant (Cao et al., 2008; Singh et al, 2018) and lipid peroxidation for evaluating heat stress tolerant (Zafar et al., 2017, Chang et al., 1994) is well known.

Therefore, the present investigation using N22 a well known heat tolerant variety (Shanmugavadeival et al., 2017; Jagadish et al., 2010) and PS2 as a heat susceptible variety (Chakrabarti et al., 2010), whereas relatively tolerant to salinity (Kumari et al., 2018) along with FL478 and MTU1010 as check varieties for salt and cold tolerance studies respectively (Walia et al., 2005; Srikanth Rahul et al., 2018) was carried out to unravel the responses under different stress conditions using morpho-physiological and biochemical approaches with a view to determine the appropriate parameters for the screening of salt, cold and heat tolerant genotypes at the seedling stage.

Material and Methods

Plant Materials and Growth Condition

The two *indica* rice genotypes, N22 and PS2 were selected for the studies in response to abiotic stresses at the seedling stage. FL478 and MTU1010 were used as check for salt and cold stress tolerance studies. The seeds of all genotypes were placed in petri dishes on moistened filter papers for germination in the dark at 28°C for 2days. Before placing for germination, healthy seeds were surface sterilized using 70% ethanol for 2min and washed thrice with sterile distilled water, followed by treatment with 0.1% $HgCl_2$ for 4min to prevent contamination and finally washed thrice with sterile distilled water. After a few days, evenly germinated rice seeds were transferred into soilrite, and the pots were transferred to greenhouse at 28°C under 16/8h of light/dark condition till the plants attain four to five leaf stage growth. We maintained 60-70% relative humidity and seedlings were watered daily.

Abiotic Stress Treatments

For salinity stress experiment, all rice genotypes (N22, PS2 and FL478) were grown in basal nutrient solution as described by Yoshida et al (1976) under controlled growth condition, and then four-five leaf stage rice seedlings were transferred into Yoshida nutrient solution containing 150mM NaCl for 7days. For the analysis of cold and heat tolerance, four to five leaf stage seedlings (N22, PS2 and MTU1010) were exposed to cold stress at 12°C in a growth chamber with 16h light/8h dark for 12days. At the same stage, heat stress was imposed at 40°C for 4days on N22 and PS2 seedlings. All the morpho-physiological and biochemical experiments were performed by maintaining three biological and three technical replicates. The leaf samples were collected from control and stress treated rice seedlings, flash freezed quickly by immersing in liquid nitrogen and stored at -80°C for further evaluation.

Morphological Index

Fresh weight of each seedling was measured with the help of electric balance (Citizen precision balance, India). Average fresh weight was calculated for each treatment. Dry weight of whole seedling was measured after drying it in an oven at 80°C until constant weight was obtained. Means were calculated for each treatment.

Analysis of Physiological and Biochemical Parameters

Photosynthetic pigments content (total chlorophyll and carotenoids) of leaf samples were estimated by dimethyl sulfoxide (DMSO) method as described by Hiscox and Israelstam (1979). The proline content of the leaves was estimated by colorimetric method as described by Bates et al (1973). Leaf sample (0.2g) was used for measuring the relative electrolyte leakage as per the method described by Sairam et al (2005). Lipid Peroxidation (MDA content) was estimated using the thiobarbituric acid (TBA) method as described by Cakmak and Horst et al (1991). The MDA level was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ using the formula: MDA level (nM) = ΔA (532nm-600nm)/1.56 X 10^5.

Results and Discussion

Effects of both Rice Genotypes under Salt Stress

N22 and PS2 along with salt tolerant check rice cultivar FL478 seedlings were found healthy in the Yoshida nutrient solution under normal growth condition (Figure 1A). At the end of 7d salt stress, N22 seedlings had exhibited more severe wilting and damaged leaves; whereas, PS2 genotype showed less wilting and leaf rolling (Figure 1B). Check genotype of FL478 showed the least leaf rolling, wilting and chlorosis compared to N22 and PS2 under 150mM NaCl treatment.

Fresh and dry weight of PS2 seedlings had significantly lower reduction (56.9% and 59.7%) when compared to N22 genotype (65.7% and 67.3%) after 7days of salt stress (Figure 2A-B). Salinity stress adversely affects fresh and dry weight of seedlings in rice (Hakim et al., 2010; Kumari et al., 2016). Reduction in seedling fresh and dry weight were also reported in rice genotypes under salt stress and these genotypes maintaining higher biomass, which are considered as salt tolerant (Zafar et al., 2015). In this study, the percentage reduction of photosynthetic pigments level was higher in the salinity stress as compared to control. Total chlorophyll and carotenoids content had severe reduction in N22 (36.1% and 24.1%) compared to PS2 (14.4% and 17.9%) (Figure 2C-D). The photosynthetic pigments in sensitive rice cultivars significantly decreased as compared to the tolerant rice cultivars under salinity stress (Moradi and Ismail, 2007; Cha-Um et al., 2009). The study of Singh et al (2018) revealed that salt sensitive cultivar MI48 showed a significant reduction in chlorophyll concentration under higher salt stress than salt tolerant cultivar CSR10 as compared with their respective control.

The effect of salt stress on electrolyte leakage was found higher in N22 (53%) than in PS2 genotype (41%) (Figure 2E). The MDA content significantly increased in N22 (3fold) than PS2 (2.5fold) (Figure 2F). Singh et al (2018) found that a salt tolerance rice cultivar (CSR10) had less electrolyte leakage and MDA content than the salt sensitive rice cultivar (MI48). The results also in agreement with the study of Sairam et al (2002) which differentiate two wheat genotypes growing at different salinity levels. In the present investigation, higher accumulation of proline was observed in PS2 (5.34 fold) than N22 (3.26 fold) under salt stress (Figure 2G). Many plant species have shown higher level proline accumulation in stress tolerant genotype than stress sensitive ones under salt stress, such as rice (Singh et al., 2018), sorghum (Jogeswar et al., 2006) and wheat (Sairam et al., 2002). Similar to our results, salt tolerant genotype Panvel-3 had shown high amount of proline accumulation compared to salt sensitive cultivars Kalarata and Karjat-3 (Kumar et al., 2007). As a salt stress verifying genotype FL478 had shown highest proline accumulation and less reduction of fresh and dry weight of seedlings, photosynthetic pigments, MDA content and electrolyte leakage compared to the other two rice cultivars (Figure 2).

Effects of both Rice Genotypes under Heat Stress

There was no notable physiological variation found between the contrasting genotypes N22 and PS2 cultivars, before exposing to heat stress (Figure 3A). After 4days of heat stress, N22 seedlings displayed very less leaf rolling, wilting and dehydration when compared to PS2 seedlings. N22 seedlings leaves were still green at the end of the heat stress treatment (Figure 3B).

Fresh and dry weight of seedlings also followed a similar variation pattern in both the genotypes under heat stress. After imposing heat stress, fresh and dry weight had significant reduction in PS2 (30.3% and 32.6%) when compared to N22 genotype (10% and 9.75%) (Figure 4A-B). Reduction in morphological indexes observed under heat and cold stresses have been reported in many plant species, such as barley (Machado and Paulsen, 2001), rye grass (Inoue et al., 2004) and wheat (Hameed et al., 2012) and these such parameters were proposed as indicators of heat and cold tolerance. There was significant variation among the two rice varieties (N22 and PS2) in photosynthetic pigments concentration under control condition. Both N22 and PS2 genotypes showed a significant gradual reduction of chlorophyll and carotenoids under heat stress at 40°C. However, PS2 showed a rapid reduction of total chlorophyll and carotenoids (39.2% and 45.7%), which was significantly at higher level than that of N22 (17.15% and 28.3%) (Figure 4C-D). Many researchers have reported that heat stress causes degradation of chlorophylls and carotenoids in various crops such as wheat (Efeoglu and Terzioglu, 2009), bent grass (Liu and Huang, 2000) and fescue (Cui et al., 2006). The study of Zafar et al (2017) found that genotypes with high photosynthetic pigments including chlorophyll a, b and carotenoids under heat stress have improved heat tolerance in rice.

Lipid peroxidation and electrolyte leakage increased under heat stress in both the rice cultivars. When exposed to heat stress, electrolyte leakage increased more in PS2 (51%) than in N22 (37%) (Figure 4E). In our study, MDA content level was significantly higher in PS2 (3.05 fold) than N22 (1.81 fold) (Figure 4F). The study of Zafar et al (2017) stated that tolerant varieties showed lower levels of damage in terms of cell membrane stability under heat stress. When exposed to high temperature, oxidative stress was frequently produced in rice seedlings, which increases the MDA content (lipid peroxidation) level which led to membrane corrosion (Hameed et al., 2012). Our findings evidently showed, among the two rice cultivars higher accumulation of proline content was observed in N22 (7.42 fold) under heat stress followed by PS2 (2.10 fold) (Figure 4G). N22 had also been reported as heat tolerant by many researchers and subsequently used in molecular dissection of heat stress response in rice (Shanmugavadeival et al., 2017; Jagadish et al., 2010). Tang et al. (2008) found decrease in proline content in rice leaves at 40°C and concluded that this response may be due to plant sensitivity to severe heat stress. Proline also involves in function as a respiration substrate (Britikov et al., 1965) this function may be responsible for the higher proline content of N22 genotype at 40°C. In effect, the rapid metabolism of this amino acid likely provides energy for mitochondrial respiration (Hare and Cress, 1997).

Effects of both Rice Genotypes under Cold Stress

There were no significant phenotypic changes among all rice cultivars, before imposing cold stress (Figure 5A). After cold stress, N22 genotype showed more leaf rolling, wilting and drying compared to PS2. MTU1010, the check variety for cold stress experiment showed slight physiological changes when compared to the other two rice cultivars (Figure 5B).

We observed significantly higher reduction of biomass (fresh and dry weight) in N22 genotype (38.6% and 39%) as compared to PS2 (22.2% and 25.6%) under cold stress (Figure 6A-B). Reduction of seedling fresh and dry weight after cold treatment can be used as morphological indicator of cold damage (Bonnecarrere et al., 2011). Comparably higher concentration of chlorophyll and carotenoids were observed in PS2 than N22 genotype under normal growth condition. The rate of photosynthetic pigments reduction was also lower in PS2 under cold stress (Figure 6C-D). Cold stress can inhibit chlorophyll synthesis and chloroplast formation in rice leaves. The study of Yadegari et al (2007) has also reported that chlorophyll content decreased in soybean seedlings, when exposed to cold stress.

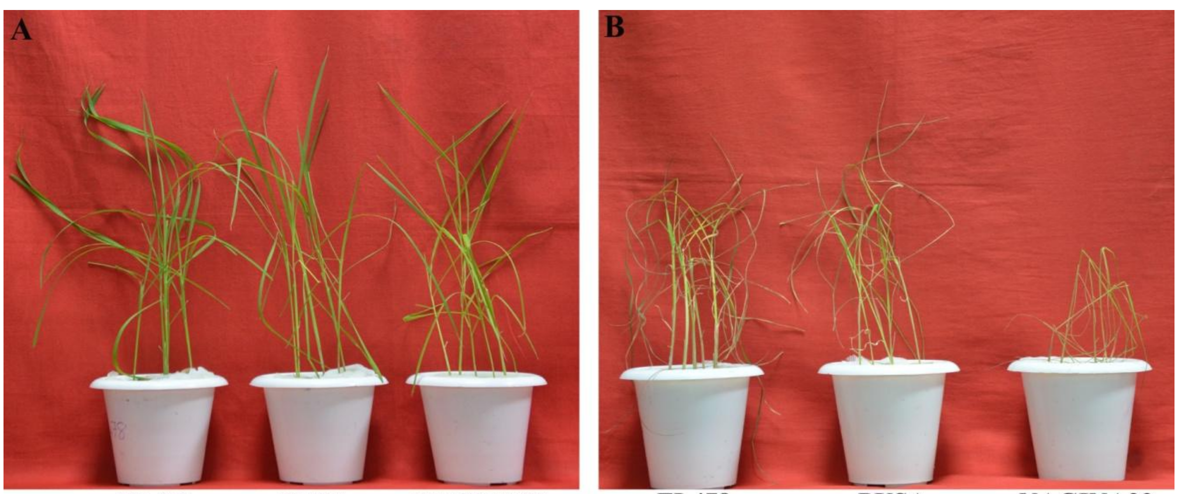
Cell membrane system is the main focus of freezing injury in plants (Hodgson and Raison, 1991). In this investigation, N22 genotype had significantly higher electrolyte leakage (55.9%) than PS2 (45.9%) (Figure 6E). As shown in figure 6F, increased lipid peroxidation was observed under cold stress in all rice cultivars. When exposed to cold stress, MDA content increased more in N22 (3fold) than PS2 genotype (2.4fold). Yamada et al (2004) worked on rye grass plants to measure the ion leakage under cold stress and observed that the genotypes with high ion leakage were sensitive to the cold stress.

Accumulation of proline content increased under low temperatures (Tamizi et al., 1995; Wang et al., 2008). In our study; we observed no significant variation for proline content between the genotypes under control condition. During cold stress, we found that accumulation of proline content level was 3.39 and 6.16 fold higher than control in N22 and PS2 genotypes, respectively (Figure 6G). A similar finding was observed in the study of Habibi et al (2011) which exhibited that Gaskoghen and Marvdasht wheat varieties had the lowest and highest accumulation of proline content, respectively. The cold tolerant check genotype MTU1010 showed very low reduction in biomass (fresh and dry weight), MDA content, electrolyte leakage, photosynthetic pigments, and higher accumulation of proline substance than the other two rice genotypes (Figure 6).

Conclusion

Response of rice seedlings to abiotic stress evaluated through morpho-physiological and biochemical attributes could be used to screening of salt, cold and heat tolerance genotypes at the seedling stage. Finally, we conclude that morpho-physiological and biochemical index is appropriate to screen for abiotic stress tolerance at the seedling stage in rice.

Figure 1:



FL478 PUSA SUGANDH2 NAGINA22 FL478 PUSA SUGANDH2 NAGINA22

Figure 2:

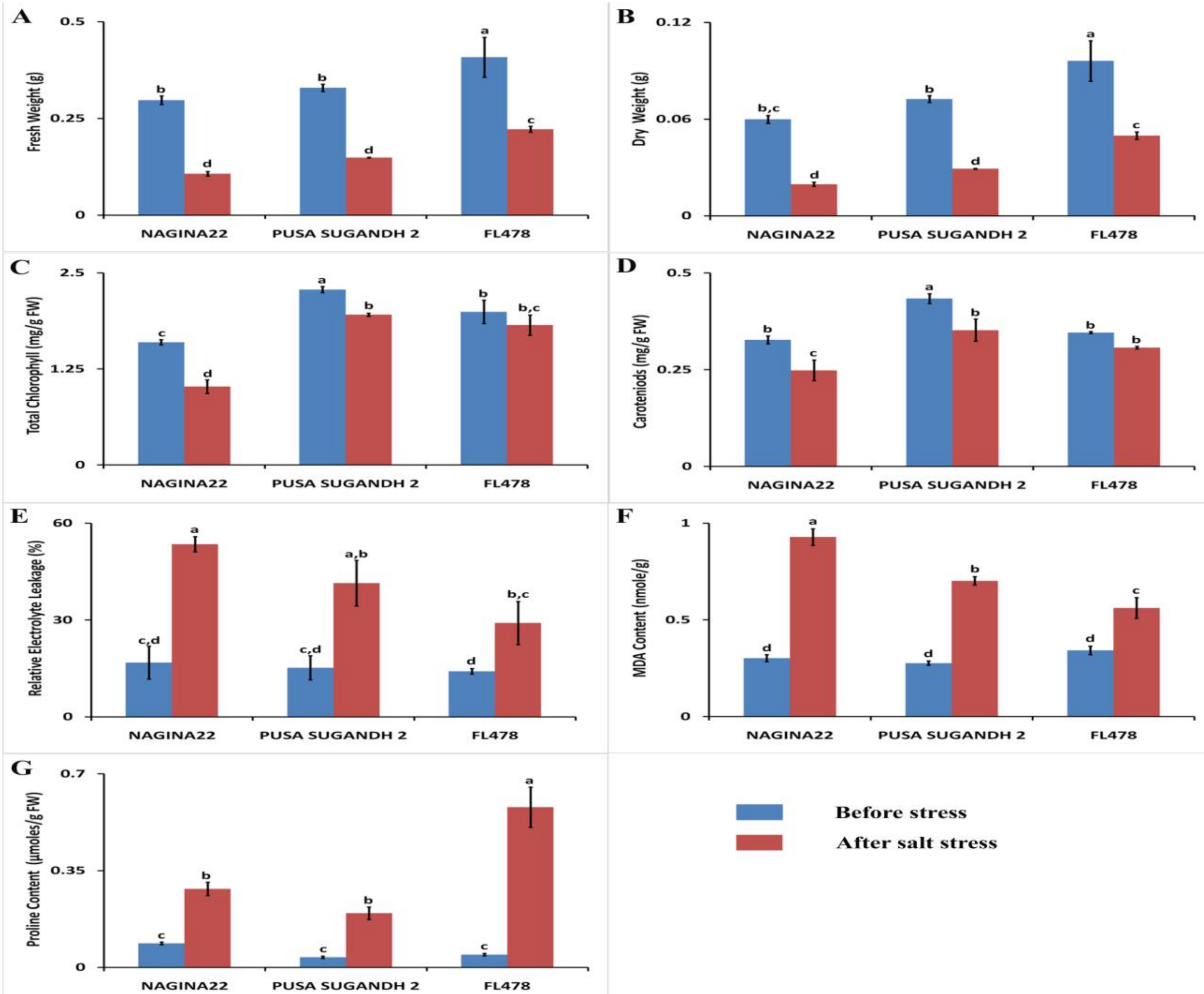


Figure 3:

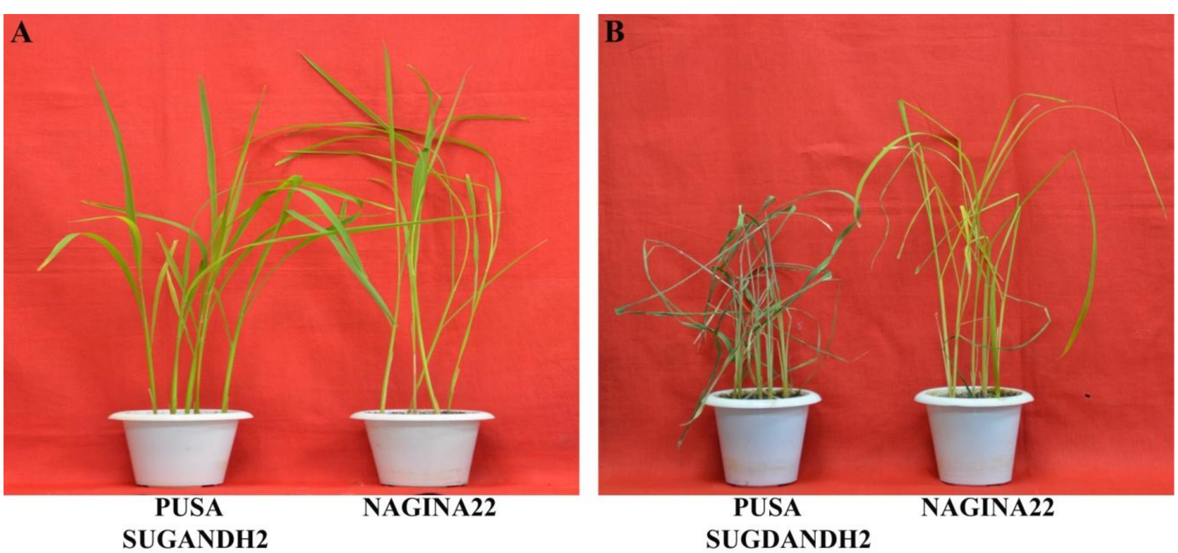


Figure 4:

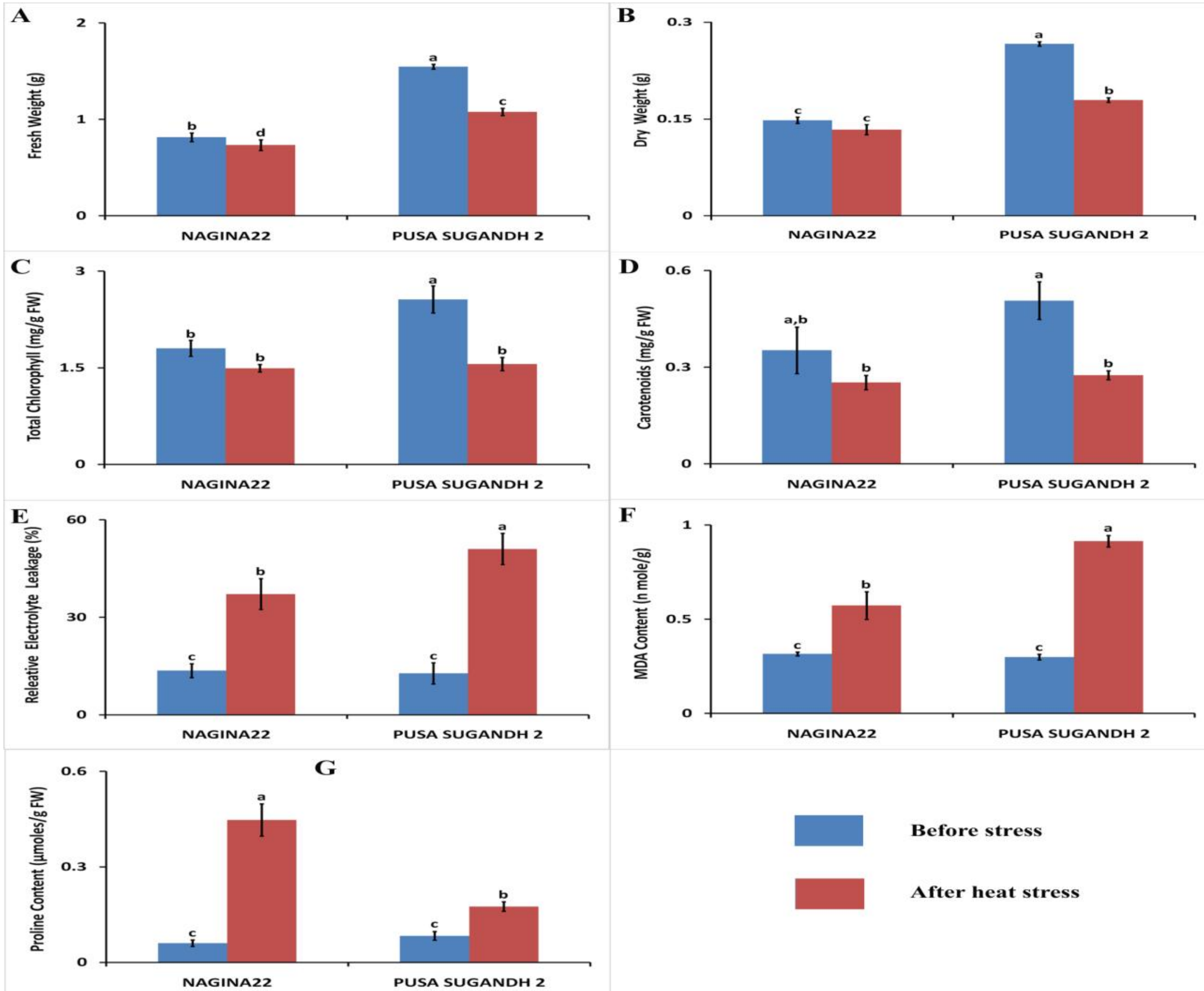
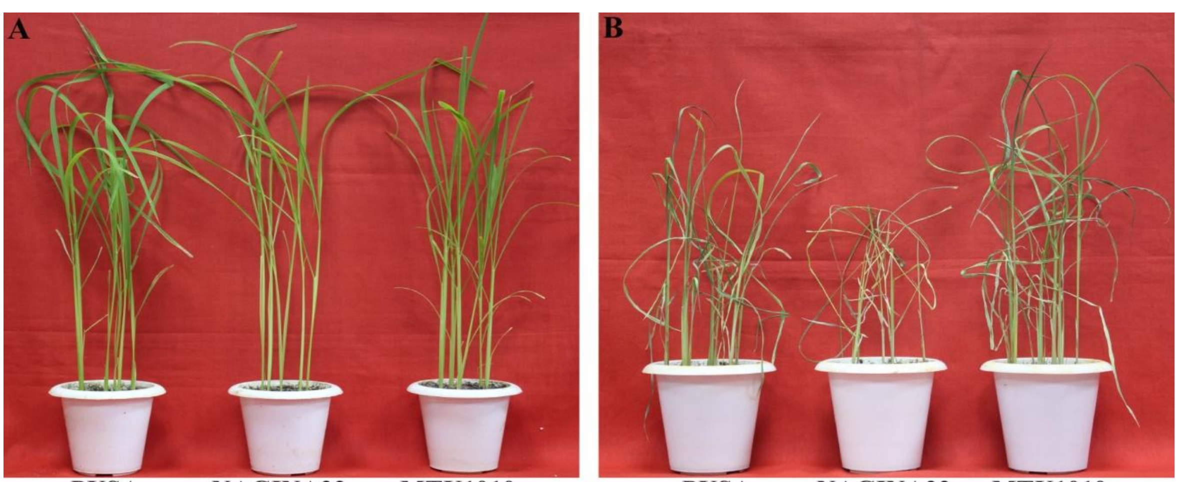


Figure 5:



PUSA SUGANDH2 NAGINA22 MTU1010 PUSA SUGANDH2 NAGINA22 MTU1010

Figure 6:

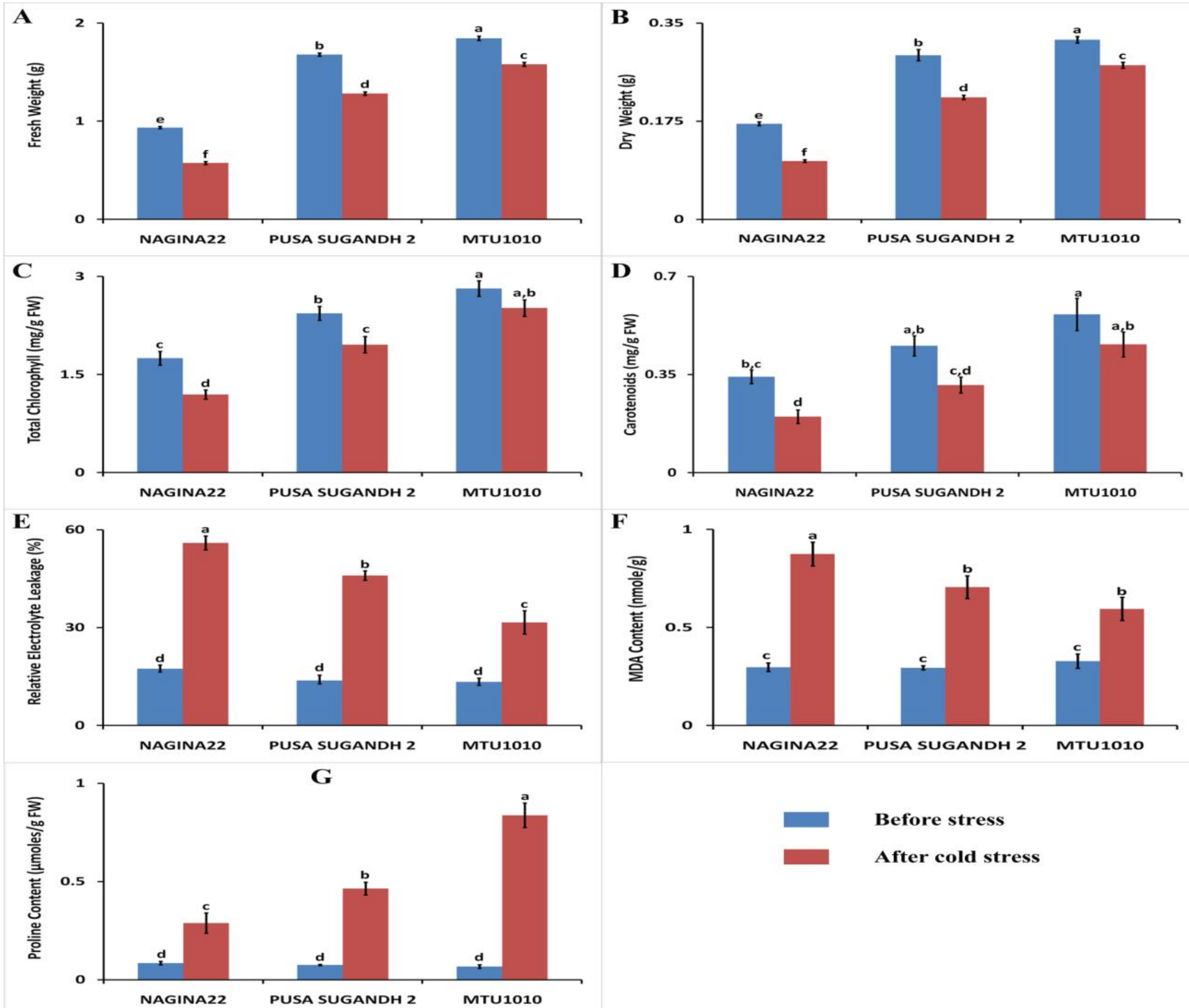


Figure Legends

Figure 1: Phenotype analysis of rice genotypes seedlings in response to salinity stress. (A) Phenotypes of 4-5th leaf stage of N22, PS2 and FL478 genotypes rice seedlings were grown on Yoshida nutrient solution, (B) Seedlings pre-cultured of 4-5th leaf stage were treated with 150mM NaCl treatment for 7days.

Figure 2: Effect of salt stress on rice seedlings morpho-physiological and biochemical parameters (A-B) Seedlings fresh and dry weight (C-D) Total chlorophyll and carotenoids content, (E) Relative electrolyte leakage (F-G) Accumulation of MDA content and proline content. Each value is the mean of three biological and three technical replicates and the vertical bars give the standard error (SE) of the

mean. Alphabets above bars indicate significant differences (p-value≤0.05) between rice genotypes.

Figure 3: Phenotypic changes of N22 and PS2 rice genotypes seedlings by heat stress. (A) Phenotype of four-five leaf stages of N22 and PS2 genotype seedlings, before heat treatment, (B) phenotype appearance of N22 and PS2 seedlings were exposed to heat stress at 40°C for 4 days.

Figure 4: Response of morpho-physiological and biochemical traits in N22 and PS2 genotype under heat stress. (A-B) Fresh and dry weight of seedlings, (C) total chlorophyll, (D) carotenoids, (E) electrolyte leakage (F) MDA content and (G) accumulation of proline content. Each value is the mean of three biological and three technical replicates and the vertical bars give the standard error (SE) of the mean. Alphabets above bars indicate significant differences (p-value≤0.05) between rice genotypes.

Figure 5: Phenotype analysis of rice genotypes seedlings in response to cold stress (A) Growth performances of N22, PS2 and MTU1010 genotypes of 4-5th leaf stage rice seedlings under normal growth condition, (B) Four to five leaf stage N22, PS2 and MTU1010 rice genotypes seedlings were cold stressed at 12°C for 12days.

Figure 6: Morpho-physiological and biochemical trait performances of genotypes under cold stress. (A-B) Fresh and dry weight of seedlings, (C) Total chlorophyll, (D) Carotenoids, (E) Relative electrolyte leakage, (F) MDA content (G) Proline content. Each value is the mean of three biological and three technical replicates and the vertical bars give the standard error (SE) of the mean. Alphabets above bars indicate significant differences (p-value ≤0.05) between rice genotypes.

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Author contributions: JK performed the experiments and drafted the manuscript. AMS helped in abiotic stress treatment and manuscript editing. KBP helped in physiological analysis and manuscript drafting. SRS helped in biochemical analysis and manuscript preparation. PKM designed the experiments, revised the manuscript, and supervised the entire work.

Conflict of Interest: The authors declare that they have no conflict of interest.

Abbreviations:

DMSO - Dimethyl sulfoxide

REL - Relative electrolyte leakage,

MDA - Malondialdehyde,

N22 - Nagina22,

PS2 - Pusa Sugandh2,

TCA - Trichloro acetic acid,

TBA - Thiobarbituric acid.

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